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14. ABSTRACT
Athletes in contact sports who have sustained multiple concussive traumatic brain injuries are at high risk for delayed, progressive neurological and psychiatric deterioration [1-9]. This syndrome is termed chronic traumatic encephalopathy (CTE) [1,7,10], and is also known as dementia pugilistica [3,11] or 'punch drunk' syndrome [9,12]. US military personnel [13,14] and others who have sustained multiple concussive traumatic brain injuries [15-17] may also be at risk for this condition. Currently, there are no methods to identify progressive tau pathology in living humans. **Hypothesis:** Aggregated forms of hyperphosphorylated tau protein formed acutely in the setting of traumatic brain injury can seed further aggregation of intracellular tau in nearby cells, leading to delayed propagation of tau pathology and neurodegeneration. **Objective:** To develop standardized, high-throughput blood and cerebrospinal fluid assays for aggregated forms of tau responsible for propagation of tau pathology after traumatic brain injury.
Progress to date: To date, none of the attempts to model progressive tau pathology after repetitive concussive TBI in mice has been optimal. Ongoing efforts include development of more sensitive methods to detect tau, and combinations of repetitive concussive TBI with binge ethanol administration. Substantial progress towards increasing the sensitivity of cell-based assays for tau aggregation activity has been made, and additional antibody-based tau detection methods for blood samples are in development.

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2) INTRODUCTION: Athletes in contact sports who have sustained multiple concussive traumatic brain injuries are at high risk for delayed, progressive neurological and psychiatric deterioration [1-9]. This syndrome is termed chronic traumatic encephalopathy (CTE) [1, 7, 10], and is also known as dementia pugilistica [3, 11] or 'punch drunk' syndrome [9, 12]. US military personnel [13, 14] and others who have sustained multiple concussive traumatic brain injuries [15-17] may also be at risk for this condition. Hyperphosphorylation and aggregation of tau protein are key pathological features of chronic traumatic encephalopathy, but at present they can only be observed post-mortem [1, 3, 6, 18-20]. Tau pathology has also been observed after single more severe traumatic brain injuries [21-23]. Currently, there are no methods to identify progressive tau pathology in living humans. The progressive aspect of chronic traumatic encephalopathy suggests that repetitive injuries may trigger an ongoing degenerative process similar to other diseases characterized by progressive tau pathology such as Alzheimer disease and frontotemporal dementia. A leading hypothesis regarding the progression of tau pathology in Alzheimer disease and frontotemporal dementia is that tau aggregates formed in one cell can propagate by exiting that cell and entering anatomically connected cells to induce tau aggregation in these cells [24-30]. While the tau pathology in chronic traumatic encephalopathy is distinct from other diseases, the propagation model offers a new conceptual framework to test these ideas in chronic traumatic encephalopathy.

The development of diagnostic tests and approaches to monitor the progression of post-traumatic tau pathology has been limited both by the lack of an appropriate small animal model and the lack of a suitable assay system. The Brody lab has recently developed a small animal model. Specifically, controlled cortical impact traumatic brain injury in 3xTg-AD and TauP301L mice, two transgenic mouse lines overexpressing mutant forms of human tau, caused tau aggregation and hyperphosphorylation in the fimbria, amygdala and hippocampi [31-33]. In parallel, the Diamond lab has recently developed cultured cell-based assays that can be used to monitor tau aggregation and trans-cellular propagation of aggregation. These assays can detect the effects of extracellular tau aggregates that cause fibril formation of native intracellular tau in cultured cells (i.e. 'seeding'), and can also track the movement of aggregates between cells [25, 26]. These advances now create an opportunity to test key hypotheses about the pathogenesis of chronic traumatic encephalopathy for the first time. In turn, a detailed understanding of this pathogenesis in experimental settings may lead to the rational development of conceptually novel diagnostic tests and therapeutic approaches in humans.

Hypothesis: Aggregated forms of hyperphosphorylated tau protein formed acutely in the setting of traumatic brain injury can seed further aggregation of intracellular tau in nearby cells, leading to delayed propagation of tau pathology and neurodegeneration.

Objective: To develop standardized, high-throughput blood and cerebrospinal fluid assays for aggregated forms of tau responsible for propagation of tau pathology after traumatic brain injury.

4

BODY
PROGRESS DURING THE REPORTING PERIOD:
TASK 1: *To assess extracts from the brains of tau transgenic mice subjected to experimental traumatic brain injury for tau aggregating activity using a cultured-cell based assay.*

Brody Laboratory
To date, we have found that all of our attempts to recapitulate tau pathology following repetitive concussive TBI (rcTBI) have been unsuccessful. Specifically, we have not detected any effect of rcTBI on immunohistochemically apparent tau pathology in either hTau or P301S tau transgenic mice 1 week, 1 month and 6 months using conventional light microscopic analyses. For example, we tested the effects of 4 closed skull concussive traumatic brain injuries spaced 24 hours apart alternating between right and left sides of the skull in 6-8 week old hTau mice [34]. Littermates were randomly assigned to either 4 injuries or 4 sham procedures, then sacrificed 7 days later. The injuries produced substantial abnormal silver staining, indicative of injury (Fig 1A-B). However, there was no change in the extent of phospho-tau immunoreactivity using the CP13 antibody (Fig. 1C-D), which recognizes tau phosphorylated at serine 202 and has been commonly used to assess human CTE pathology [35]. Tau knockout mice that were littermates of the hTau animals were also assessed as negative controls. There was no difference in the extent of silver staining between injured hTau mice and injured tau knockout mice (Fig 1B).
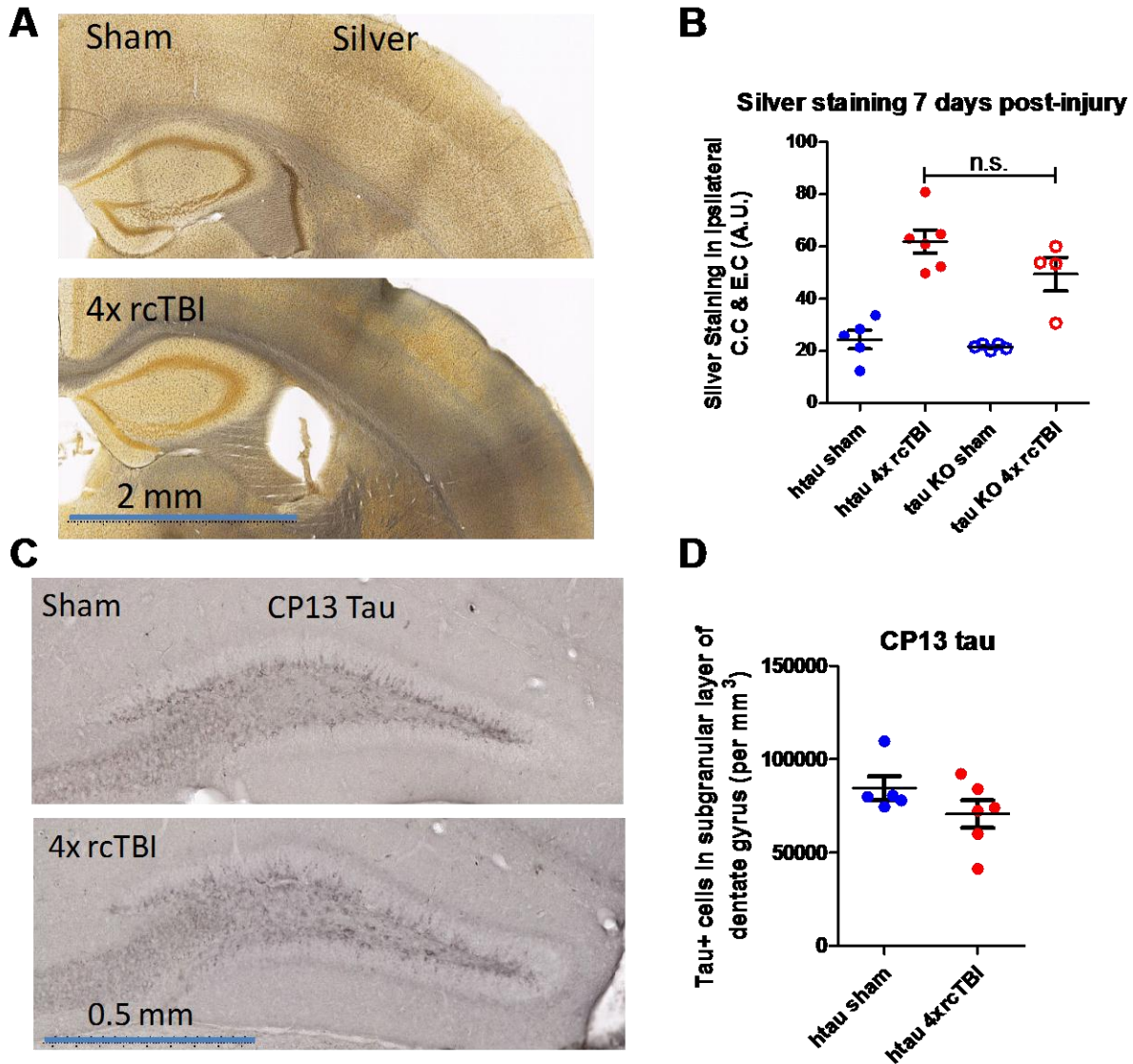
Figure 1: <u>No effects of repetitive concussive TBI on tau immunostaining in hTau mice:</u> 6-8 week old hTau mice or tau knockout littermates were injured with 4 concussive impacts 24 hours apart, then sacrificed 7 days later. **A.** The injuries caused extensive silver staining in the cortex, corpus callosum (C.C.), external capsule (E.C.) and thalamus comparable to the results previously shown for 2 concussive impacts [36]. **B.** Quantification of silver staining in the white matter by blinded observers by densitometry revealed substantially increased staining in injured mice compared with shams (p<0.001, 2-way ANOVA followed by Tukey post-hoc test. However, there was no difference in silver staining between the injured hTau mice and identically injured tau knockout littermates (A.U.: arbitrary units). **C.** Tau immunohistochemistry using CP13, a monoclonal antibody recognizing phosphorylated tau. No difference was apparent in the dentate gyrus between sham and injured hTau mice. **D.** Quantitative unbiased, blinded stereological analysis of CP13 positive cells in the subgranular layer of the dentate gyrus. No difference was found between groups (p=0.19, Student's t-test).

There are several possible non-mutually exclusive explanations for these negative results including insufficiently sensitive methods, latency from injury to development of tau pathology longer than the reasonable lifetime of mice, protective effects of anesthesia used during

experimental injury, and a requirement for additional pathophysiological events or genetic factors.

    We recognize that in mice, injured axons and potentially intraaxonal accumulation of pathological tau may be difficult to resolve by light microscopy due to the small size of mouse axons. Thus, developing methods to efficiently assess non-dilated, injured axons following rcTBI has become a top priority. We considered several approaches: (Table 1) and settled on array tomography-based immunofluorescent assessments as the most immediately promising. Array tomography involves cutting ribbons of serial ultrathin sections (70 nm thick) using an ultramicrotome, then performing immunofluorescent labeling on the ribbons (Micheva and Smith, 2007). The ultrathin sections provide exceptionally good signal to noise and spatial resolution, substantially better than optical (e.g. confocal) sections. The method has been used to quantify synaptic loss in both mouse models and human tissue (Koffie et al., 2012; Koffie et al., 2009). We adapted this technique to the study of injured white matter following repetitive concussive TBI by using the monoclonal antibody SMI32, which works well in resin-embedded sections and stains only injured axons in the mouse brain. Using largely automated image processing techniques, we determined that mouse corpus callosum contains approximately 2,000 SMI32- immunoreactive axons per cubic mm 7 days after repetitive concussive TBI (vs. essentially zero in control mice and in conventional thick sections stained with APP). Co-labeling with tubulin antibody in the same sections allowed us to determine that there were approximately 20,000 total axons per cubic mm in the region, and that the total number was not diminished by rcTBI at this time point (Bennett et al, J Neuroscience Methods, *in press*).

Table 1: Approaches to assessing non-dilated injured axons following rcTBI

	Advantages	Disadvantages
Quantitative Electron Microscopy	Highest standard of sensitivity May provide insight into mechanisms (myelin damage, cytoskeletal disruption…)	Slow, labor intensive, expensive, prone to fixation artifacts, challenging to combine with molecular specific assessments Small regions assessed Manual counting of axons required
Silver staining of conventional tissue sections	Relatively fast and inexpensive Large regions of tissue assessed	Unknown mechanism of staining Nonspecific Not fully quantitative
Superresolution fluorescence microscopy	Potentially high sensitivity Potential to provide insight into mechanisms through molecular targeted probes.	Slow Very expensive Small regions assessed Unknown artifacts Will require extensive validation
Array tomography-based immunofluorescence microscopy	More sensitive than conventional fluorescent microscopy Faster than electron microscopy and superresolution microscopy Molecularly targeted probes Larger regions assessed than EM or superresolution microscopy High signal to noise allowing automated counting of injured axons	Unknown sensitivity to the smallest injured axons More labor intensive than conventional light microscopy Approximately 50% of antibodies work well in resin-embedded ultrathin sections.

Preliminary data indicate that we can detect tau using array tomography (Figure 2). The tau antibodies PHF1, AT8, and CP13 were tested in tissue from aged mice carrying a familial frontotemporal dementia mutation (TauP301S) as a positive control. These phospho-tau antibodies all detected punctate aggregates in entorhinal cortex (PHF1 shown, Figure 2 D-F). No tau labeling was seen in the cortex of tau knockout mice (PHF1 shown, Figure 2 D inset).

Next we asked whether tau accumulation was detectible following experimental TBI in mice. To date, we have seen sparse and morphologically indistinct tau labeling in 3xTg-AD mice at 7 days following 4 rcTBIs (Figure 3). Labeling was performed using human polyclonal anti-human tau (htau) antibody directly labeled with Alexa 568 (bottom left panel). Background fluorescence was high. Injured axons labeled with SMI32 were readily apparent (upper right panel), but did not appear to colocalize with tau staining. Tau and SMI32 staining in sham (uninjured) young 3xTg-AD mice was minimal (Figure 4). Thus, while promising, the array tomography method requires further optimization for assessment of tau pathology following rcTBI.
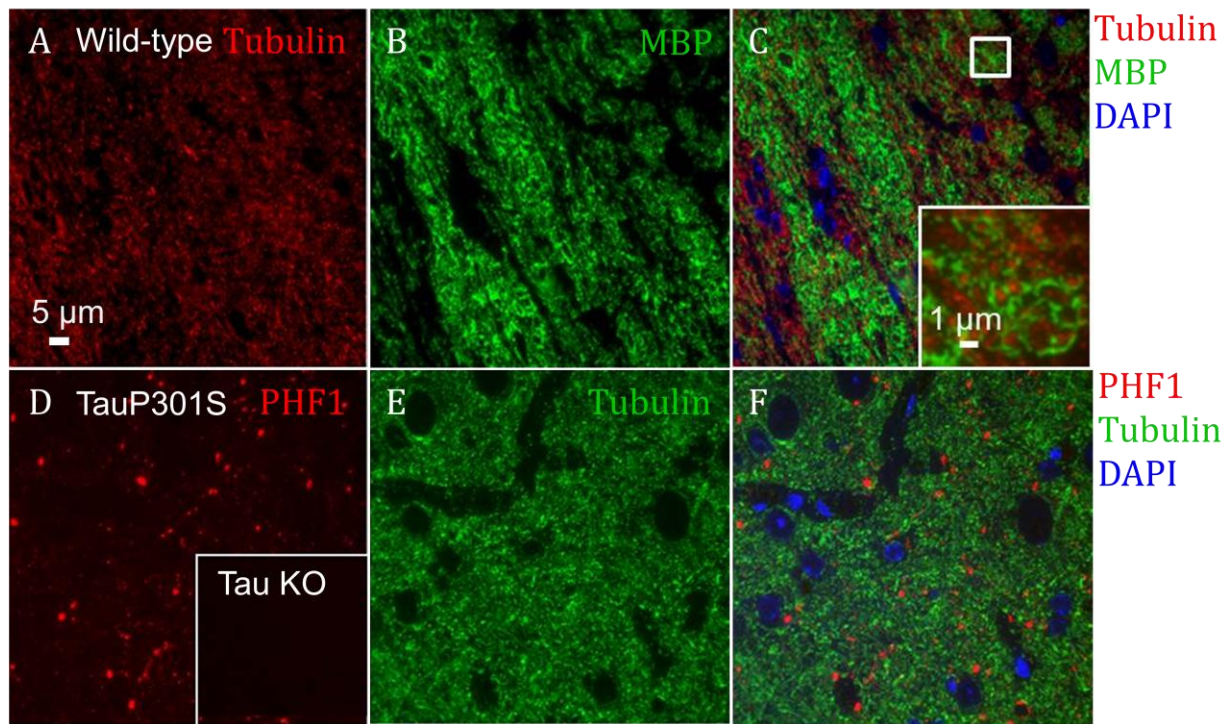

Figure 2. Detection of myelin basic protein and tau using array tomography. **(A-C)** Myelin basic protein and tubulin labeling in the external capsule of an uninjured wild-type mouse. (A) Tubulin-Alexa 594 labeled axons. (B) Myelin basic protein (MBP)-Alexa 488 labeled axons. (C) Composite image of DAPI, myelin basic protein, and tubulin. Inset shows an enlarged view of the box in (C), where individual myelinated axonal cross-sections are clearly visible. **(D-F)** PHF-1 tau and tubulin labeling in the entorhinal cortex of an uninjured 12-month-old transgenic Tau P301S mouse expressing a mutant form of tau implicated in frontotemporal dementia. (D) PHF1-Cy3 labeled punctae. Inset shows the absence of PHF1 labeling in cortex from a tau knockout mouse. (E) Tubulin Alexa 488 labeled neuropil. (F) Composite image of DAPI, tubulin, and PHF1.
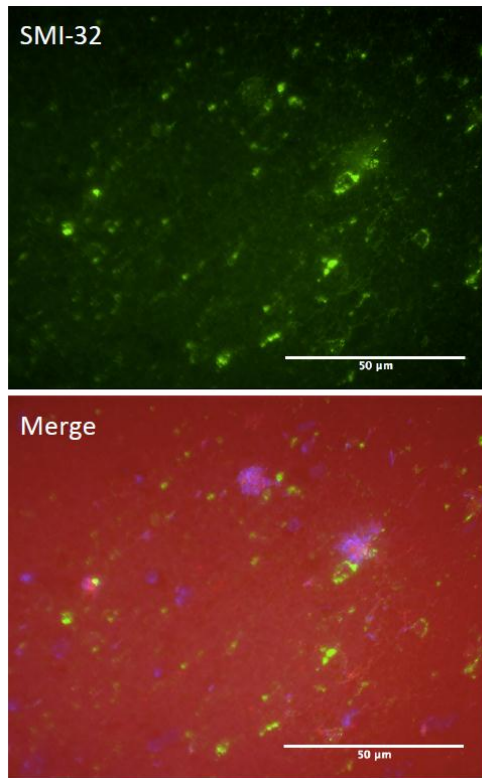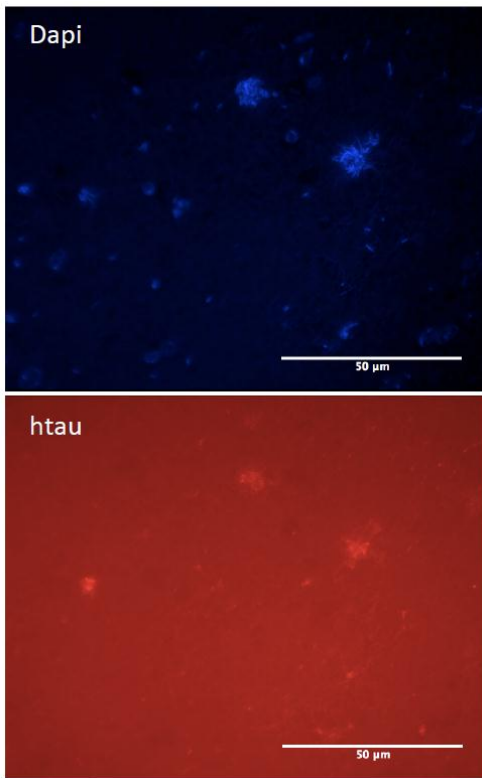
Figure 3: <u>Nonspecific tau labeling using array tomography 1 week after 4 repetitive concussive TBI's in 3xTG-AD mice.</u> Upper left: DAPI staining for nucleu. Upper right: SMI32 staining for injured axons (as in Bennett et al JNeuroscience Methods, *in press*). Lower left: polyclonal anti-human tau (htau) staining. Note high background and morphologically indistinct staining. Lower right: merger of all 3 images.
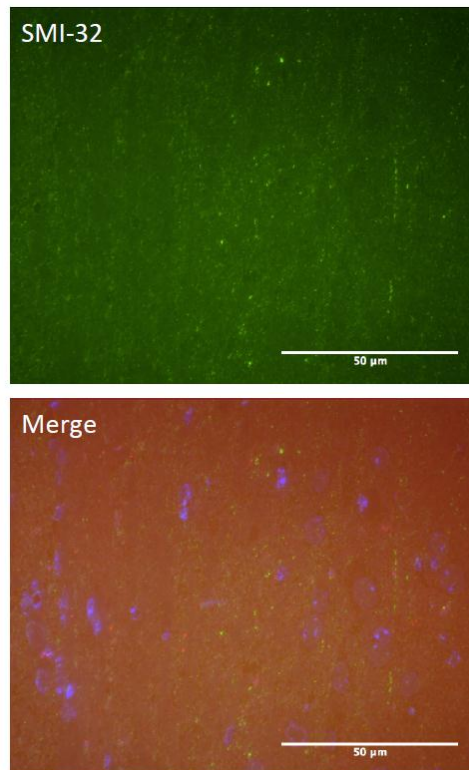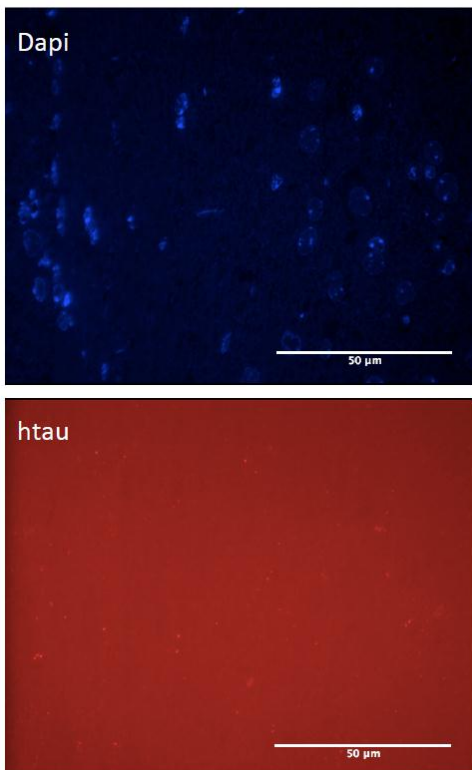

Figure 4 <u>Tau labeling using array tomography 1 week after 4 sham procedures in 3xTG-AD mice.</u> Minimal SMI32 and tau labeling in the absence of injury.

It is apparent from the clinical experience of Dr. Brody and colleagues who care for retired professional athletes and military personnel that not all of these men who have sustained multiple concussive traumatic brain injuries go on to develop progressive neurological and behavioral deterioration (unpublished observations). Many of them are cognitively normal and have good emotional balance. Others have deficits in cognitive function and emotional regulation that are static rather than progressive. Thus, a critical question is why some individuals appear resilient while others are more vulnerable to progressive deterioration following multiple concussive TBIs. In general, we are interested in determining in both a clinical and experimental setting whether additional factors such as alcohol abuse, anabolic steroids, narcotics, sleep deprivation, sleep apnea, systemic injuries and inflammatory states contribute to axonal injury, tau pathology, and progressive neurological deterioration. Our conceptual model is that after injury, there may be two competing processes: clearance of pathological tau and trans-synaptic propagation of pathological tau. If trans-synaptic propagation of pathological tau species overwhelms clearance, progressive tau pathology and possibly neurological deterioration may result. Instead, if clearance prevails, the model would predict that deficits would recover over time or remain static, since some deficits are likely due to other, non-tau related pathology such as severed axons or neuronal cell loss.

Specifically, we have initiated experiments to test the hypothesis that the interaction between rcTBI and binge alcohol use promotes tau aggregation and neurodegeneration. The design of the experiment, initiated in October 2014, is shown in Table 2.

Table 2: Experimental design for interaction between rcTBI and binge alcohol use

Genotype	Injury	Ethanol Administration	Note
hTau +/+	5x rcTBI	20% in drinking water for 3 hours during dark phase	Hypothesis: most severe tau pathology and degeneration
hTau +/+	5x rcTBI	None (water bottle changes only)	Hypothesis: less severe or no tau pathology, less severe degeneration
hTau +/+	5 shams	20% in drinking water for 3 hours during dark phase	Control for the effects of ethanol alone on tau pathology and neuro-degeneration
hTau +/+	5 shams	None (water bottle changes only)	Control for the effects of aging and handling in hTau mice.
Tau -/-	5x rcTBI	20% in drinking water for 3 hours during dark phase	Assessment of the role of tau pathology, if any, in combined rcTBI + ethanol induced neurodegeneration and inflammation
Tau -/-	5x rcTBI	None (water bottle changes only)	Assessment of the role of tau in late rcTBI induced neurodegeneration and inflammation
Tau -/-	5 shams	20% in drinking water for 3 hours during dark phase	Control for the effects of ethanol alone on neurodegeneration in the absence of tau
Tau -/-	5 shams	None (water bottle changes only)	Control for aging and handling alone.

We are using hTau mice expressing normal (nonmutated) human tau under endogenous regulatory control. This line has the highest face validity as a model of human tau-related neurodegeneration, in our view since it does not involve mutated or overexpressed tau. The injury model was changed based on the recent findings from the Crawford lab to involve 5 closed skull midline injuries at 48 hour intervals [37, 38]. The binge ethanol protocol was adopted from prior published studies indicating that mice will consume substantial quantities of ethanol in the early part of the dark phase if it is gradually introduced into their drinking water over a 2 week period [39]. Alcohol administration is being given 6 days per week over a 6 month period. Mice are scheduled for sacrifice and blinded stererological assessment for tau pathology, axonal degeneration, brain atrophy, and neuroinflammation in March, 2015

Diamond Laboratory
We have not yet detected seeding activity in existing mouse models of TBI. However, the Brody laboratory is making great progress in the analyses of mouse models of repetitive concussive injury. The cell-based assay we have developed is likely to be very useful in detecting seeding activity (Holmes, PNAS 2014). In related work not funded by this contract, we have successfully used the cell-based assays to measure tau pathology in human brains from patients with tauopathy neurodegeneration syndromes. Thus, when should mouse models produce seeding activity in brain following injury, we expect to detect it.

TASK 2: *To determine whether mouse blood and cerebrospinal fluid tau aggregating activity quantitatively predict brain tau pathology and neurodegeneration in mice subjected to experimental traumatic brain injury*

Brody Laboratory
We have not performed any further tests of mouse blood or cerebrospinal fluid, since the optimal model for rcTBI-related tau pathology has not been determined.

Diamond Laboratory
In our prior progress report, we described data for an ultra-sensitive cell-based assay that is ready to be deployed to study mouse models of TBI. We have evaluated current mouse models of TBI and have not found trauma-induced tau seeding activity in the cell-based assay. To solve this problem, we wish to study mice that produce robust tau pathology exclusively in brain via Cam-K Tet-off x TET-P301L, so that we can develop blood-based assays to detect tau. Transgenic models existing in the lab do not have this feature, and are thus contaminated by tau in the periphery. The mouse line we are using (Tg4510) only became available from Jackson Labs in the fall of 2014. We obtained the breeders, and have been creating lines suitable for evaluating our cell-based assays. These animals are still being bred for experiments (they are very slow breeders), and it is likely to take several months to generate the animals required for our studies. Our goal here is to create a model with brain-specific tau expression (as opposed to other extant tau models) so that we can still optimize a tau aggregate detection assay, even if we do not have an ideal mouse model.

TASK 3: *To test whether antibodies that block tau aggregating activity in cultured cell-based assays also block tau pathology, neurodegeneration and behavioral deficits in mice subjected to experimental traumatic brain injury*

Brody and Diamond Laboratories

We have not made progress on this aim, because we are still working out the parameters of the experimental mouse models of TBI. Until we have a robust mouse model of TBI it will not be feasible to test antibody therapies.

TASK 4: To develop an antibody-based assay for tau aggregating activity

Brody Laboratory

We have not performed any experiments related to antibody-based assays during this award period.

Diamond Laboratory

It is our anticipation that tau pathology in TBI patients is going to be distinct from other types of tau pathology, e.g. that associated with Alzheimer's disease, because of the different routes to its formation (trauma vs. aging) and the different neuropathological appearances of the tau pathology in the brain. A major challenge will be to discriminate these different forms of tau as we attempt to parse TBI pathology from other forms of tau pathology. We have thus developed a "sandwich" antibody detection system to monitor for the presence of tau aggregates. This system has the power to discriminate different types of tau fibrils, and is ready to be deployed in animal models of tauopathy, including TBI. When the mouse models come on line for use we will be ready to test it. The assay is based on the fact that when a monoclonal antibody is coupled to a bead, secondary antibody that is identical to the first will bind only if a multimer of tau has bound the first antibody (due to replication of epitopes). Monomer does not produce a signal.
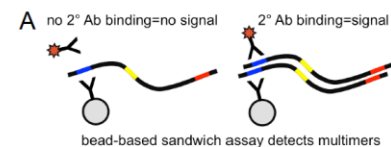

**Figure: A multiplex avidity profile to detect aggregates.**
(A) Nanoparticles are coated with a specific monoclonal antibody, and an identical antibody is used as a detection reagent. When monomer has bound the particle, the secondary antibody cannot bind. Only when multimers of tau have bound does the secondary antibody bind and produce a signal. (B) When tau fibrils form that take on distinct conformations, epitopes will be made differentially available to individual monoclonal antibodies. This can allow a "barcode" to be determined based on the sandwich signal from each of multiple antibodies. In the cartoon, only three antibodies are used. The barcodes can be plotted in multivariate space to characterize antibody conformation, and then compared using a variety of statistical algorithms.
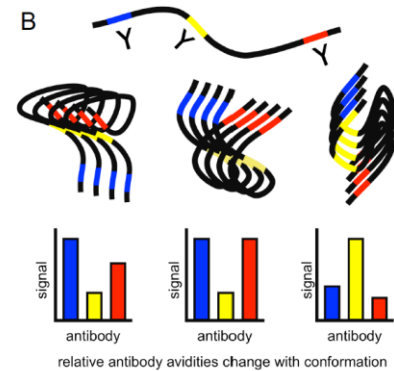
By fibrillizing tau under different conditions, we created "test samples" that consist of fibrils of distinct conformation. These were then tested using 5 different anti-tau monoclonal antibodies, in which each was used in its own sandwich format. This produces 5 independent antibody avidity

profiles, which enable a "barcode" to be formed for each conformation of tau. We assessed these conformations in triplicate. The assay was readily able to "bin" different preparations of tau based solely on their conformation.

Figure: A multiplex avidity assay to assess the composition of tau fibrils. The use of multiple antibodies in a sandwich format allows profiling of aggregates to compare their global structure. (A) We used 5 antibodies that target distinct epitopes of tau. Each antibody was used to study three different tau fibril conformers. (B) The three fibrillar conformers (A,B,C) were each studied in triplicate. Each bar color represents a different antibody. Note that the antibodies recognize the conformers with distinct avidities. This allows a "profile" of binding to be determined. (C) Euclidean distance was used to compare the signals of different fibrillar species in a pairwise comparison. Note that each fibrillar conformation clusters with similar preparations, and is distinct from the other conformations. This indicates that the multiplex avidity profile can distinguish different aggregate conformations. We anticipate using this assay to monitor the production of distinct tau aggregates within the brains of tauopathy mice and, ultimately, patients.
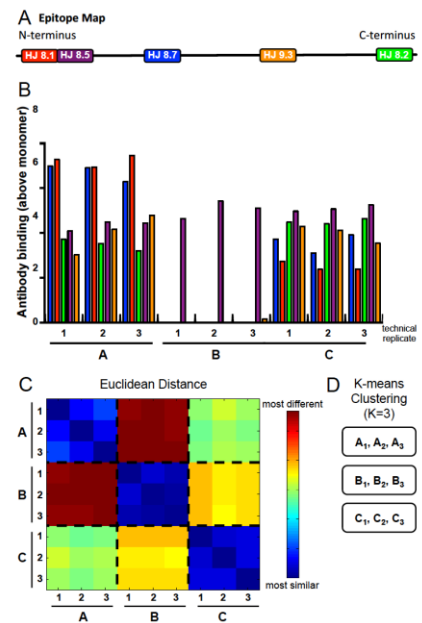
4) **PROBLEM AREAS**: We continue to work to develop mouse models of TBI that recapitulate individual aspects of human TBI-related neurodegeneration, including progressive tau pathology, atrophy, and tau seeding activity. This will be important for validation of therapeutic approaches. In the near term, we are adapting existing mouse models of pathology (Tg4510) to enable us to search for tau in the periphery that can only derive from CNS tau pathology. We are developing methods to increase the yield of aggregate detection in the periphery. We will be testing whether peripherally administered antibodies can trap tau in the blood, and might thus increase the likelihood of detection of tau seeds.

5) **WORK TO BE PERFORMED IN THE NEXT PERIOD**: During the next 3 months we expect to perform the following:

Brody Laboratory
We plan to complete the rcTBI + ethanol experiments outlined in Table 2 during the next 3 months. After sacrifice of the mice, half of the brain will be dissected, frozen and used for biochemical studies of tau aggregation, seeding activity, and oligomerization. The other half will be fixed and assessed using immunohistochemical staining for tau pathology (total tau, CP13, AT8, PHF1), microgliosis (Iba1), and neurodegeration (white matter, hippocampal and thalamic volume loss by Cavalieri method, hippocampal cell counts on cresyl violet stained sections). All assessments will be performed in a blinded fashion.

If successful, we will initiate an attempt to internally replicate the experiment, in accordance with good scientific principles. As part of the replication attempt, blood will be drawn after each injury, then monthly during the binge ethanol phase. The blood will be banked and tested when the optimal tests from the Diamond lab have been determined. At sacrifice, CSF will be drawn as well.

Furthermore, we plan to continue to optimize array tomography-based assessments of tau pathology in non-dilated injured axons. Additional antibodies and blocking approaches to reduce background will be systematically assessed.

Diamond Laboratory

We will continue to breed our colony of mice that express tau, and develop pathology, exclusively in the brain. These mice (Tg4510) express P301L mutant full-length human tau driven by the tetracycline promoter coupled with CamK-driven tet factor. The Diamond lab is now moved to the University of Texas, Southwestern Medical Center. Additionally, we will continue to refine the microfluidic-based detection methods in an effort to increase sensitivity. We will be optimizing methods for tau retrieval from the periphery, in anticipation of developing assays based on antibody-mediated trapping of brain-derived tau in the blood.

ADMINISTRATIVE COMMENTS:
The Diamond laboratory has moved to the University of Texas, Southwestern Medical center. However, there is an active TBI research program going on there that will help augment the lab's efforts. Drs. Diamond and Brody will continue to work closely by regular teleconference and face-to-face meetings when appropriate. Because Dr. Diamond has students who are still linked to Washington University, for the next 2-4 years he will be traveling to St. Louis periodically for the purpose of thesis committees and examinations, and thus will have additional opportunities to meet with Dr. Brody in person.

KEY RESEARCH ACCOMPLISHMENTS

1) Determination that initially proposed approaches to modeling repetitive concussive traumatic brain injury-induced tau pathology are not likely to yield a useful animal model for the development and validation of fluid biomarkers.
2) Initiation of experiments to test more sophisticated approaches including more sensitive tau pathology detection methods and combined injury + binge ethanol models.
3) Development of an antibody-based test for tau aggregates that involves the same monoclonal antibody used to capture and detect tau in an ELISA plate-based format
4) Development of antibody-coated nanoparticle based profiling for distinct conformations of tau aggregates.

REPORTABLE OUTCOMES
None

CONCLUSION
While the initial investigations performed have not turned out as expected, we are making substantial progress and have several innovative new directions planned for the next year. The problem of diagnosis and serial assessment for tau-related pathology after repetitive concussive TBI is a critical one, and continued intensive study of the topic is warranted.

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APPENDICES

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